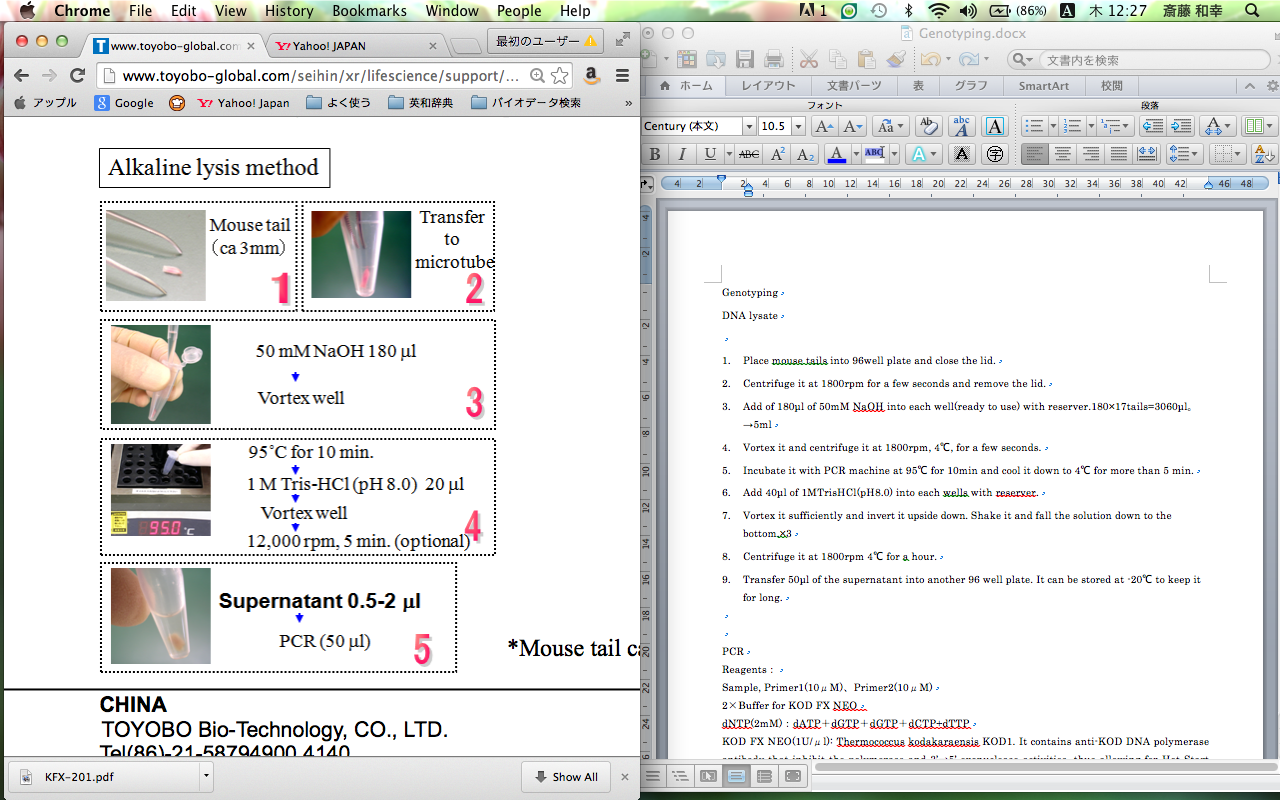
Genotyping

DNA lysate



1. Place mouse tails into 96well plate and close the lid.
2. Centrifuge it at 1800rpm for a few seconds and remove the lid.
3. Add of 180μl of 50mM NaOH into each well(ready to use) with reserver.180×17tails=3060μl。→5ml
4. Vortex it and centrifuge it at 1800rpm, 4℃, for a few seconds.
5. Incubate it with PCR machine at 95℃ for 10min and cool it down to 4℃ for more than 5 min.
6. Add 40μl of 1MTrisHCl(pH8.0) into each wells with reserver.
7. Vortex it sufficiently and invert it upside down. Shake it and fall the solution down to the bottom.×3
8. Centrifuge it at 1800rpm 4℃ for a hour.
9. Transfer 50μl of the supernatant into another 96 well plate. It can be stored at -20℃ to keep it for long.

PCR

Reagents：

Sample, Primer1(10μM)、Primer2(10μM)

2×Buffer for KOD FX NEO

dNTP(2mM)：dATP＋dGTP＋dGTP＋dCTP+dTTP

KOD FX NEO(1U/μl): Thermococcus kodakaraensis KOD1. It contains anti-KOD DNA polymerase antibody that inhibit the polymerase and 3’→5’ exonuclease activities, thus allowing for Hot Start PCR, KOD FX Neo generates blunt-end PCR products because of its 3’→5’ exonuclease (proof-reading) activity

Methods：

1. Incubate the reagents except for KOD FX NEO at 37℃.
2. Vortex the reagents and spin them down.
3. Make the master mix. Add 2×Buffer→dNTP→Primer1,2→KOD FX NEO into the 1.5ml eppendorf. Vortex it and spin it down.
4. Dispence the mastermix into each well of 96well plate. Place the droplet below line of tube.
5. Place the DNA solution above line of tube.
6. Close the lid of 96 well plate. Vortex the plate and centrifuge at 1800rpm for a few seconds. Place the plate on the thermal cycler.

Cycle condition:

|  |  |  |  |
| --- | --- | --- | --- |
|  | USAG-1 | C/EBPβ | Runx2 |
| Predenature | 94℃ 3min | 94℃ 3min | 94℃ 3min |
| Denature | 98℃ 10sec | 98℃ 10sec | 98℃ 10sec |
| Annealing | 64℃ 1min | 64℃ 1min | 57℃ 1min |
| Extension | 68℃ 1min | 68℃ 2min | 68℃ 1min |
| Cycles | 36 | 36 | 36 |
|  | 68℃ 5min | 68℃ 5min | 68℃ 5min |

Product size:

|  |  |  |  |
| --- | --- | --- | --- |
|  | USAG1 | C/EBPβ | Runx2 |
| WT | 322bp | 650bp | 528bp |
| Mt | 492bp | 1300bp | 900bp |

Master mix(μl):

USAG1：Multiplex 3＋2

|  |  |  |
| --- | --- | --- |
|  | ×1  USAG1 | ×5  USAG1 |
| 2×buffer | 5 | 25 |
| dNTP | 2 | 10 |
| DCR7A | 0.3 | 1.5 |
| DCR7B | 0.3 | 1.5 |
| LacZ Rev | 0.3 | 1.5 |
| KOD FX NEO | 0.1 | 0.5 |

C/EBPβWT：48＋5

|  |  |  |
| --- | --- | --- |
|  | ×1  C/EBPβWT | ×53  C/EBPβWT |
| 2×buffer | 5 |  |
| dNTP | 2 |  |
| 10μM Primer Fw F1 | 0.3 |  |
| 10μM Primer Rv R1 | 0.3 |  |
| KOD FX NEO | 0.1 |  |

C/EBPβMt：48＋5

|  |  |  |
| --- | --- | --- |
|  | ×1 | ×53 |
| 2×buffer | 5 |  |
| dNTP | 2 |  |
| 10μM Primer Fw NF11 | 0.3 |  |
| 10μM Primer Rv NF neo | 0.3 |  |
| DMSO | 0.5 |  |
| KOD FX NEO | 0.1 |  |

Runx2:multiplex:48＋5

|  |  |  |
| --- | --- | --- |
|  | ×1 | ×53 |
| 2×buffer | 5 |  |
| dNTP | 2 |  |
| 10μMPrimer a | 0.3 |  |
| 10μMPrimer b | 0.3 |  |
| 10μMPrimer c | 0.3 |  |
| DMSO | 0.5 |  |
| KOD FX NEO | 0.1 |  |

Reaction solution(μl):

|  |  |
| --- | --- |
| DNA | 2.5 |
| Master mix | 7.5(C/EBPβMt8) |
| Total | 10(C/EBPβMt10.5) |

Electrophoresis:

1. Wash the electrophoresis apparatus well, and add TAE into the apparatus up to the higher fluid level than the EtBr agar.

②Add 2.5μℓ of 10×Loading Buffer into 10μl of thePCR product.　※It requires the quantitiy of loading Buffer in order to precipitate DNA definitely. Trasfer 5μl of the mixture into the wells of EtBr agar.

* Loading Buffer ‘s glycerol and sugar need to give density to DNA and precipitate DNA in the wells. It’s BPB(Bromophenol blue),XC(Xylene cyanol FF) need to be in visual contact with the bands during electrophoresis.

③Place 10μl of 1kbLadder into the edge of wells.

④Electrophorese them at 100V for 15 min.

※Longer time results in wider the gap of Ladder.

⑤Check if the apparatus babbles up the bands move. ※DNA is minus charge.

⑤Observe the results with transilluminator.

※Check if the sizes of bands are the same. Pipetting and inadequate neutralization during lysis are suspected as a cause.

